Regulation of Expression of CEBP Genes by Variably Expressed Vitamin D Receptor and Retinoic Acid Receptor in Human Acute Myeloid Leukemia Cell Lines

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Regulation of Expression of *CEBP* Genes by Variably Expressed Vitamin D Receptor and Retinoic Acid Receptor α in Human Acute Myeloid Leukemia Cell Lines

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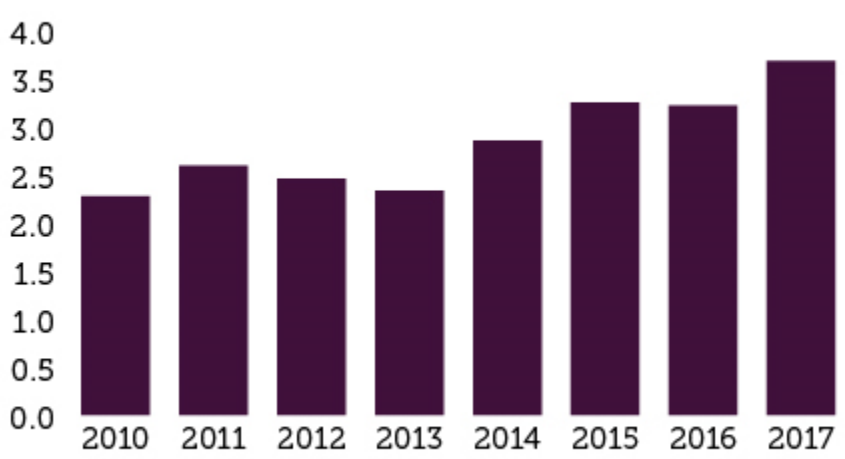

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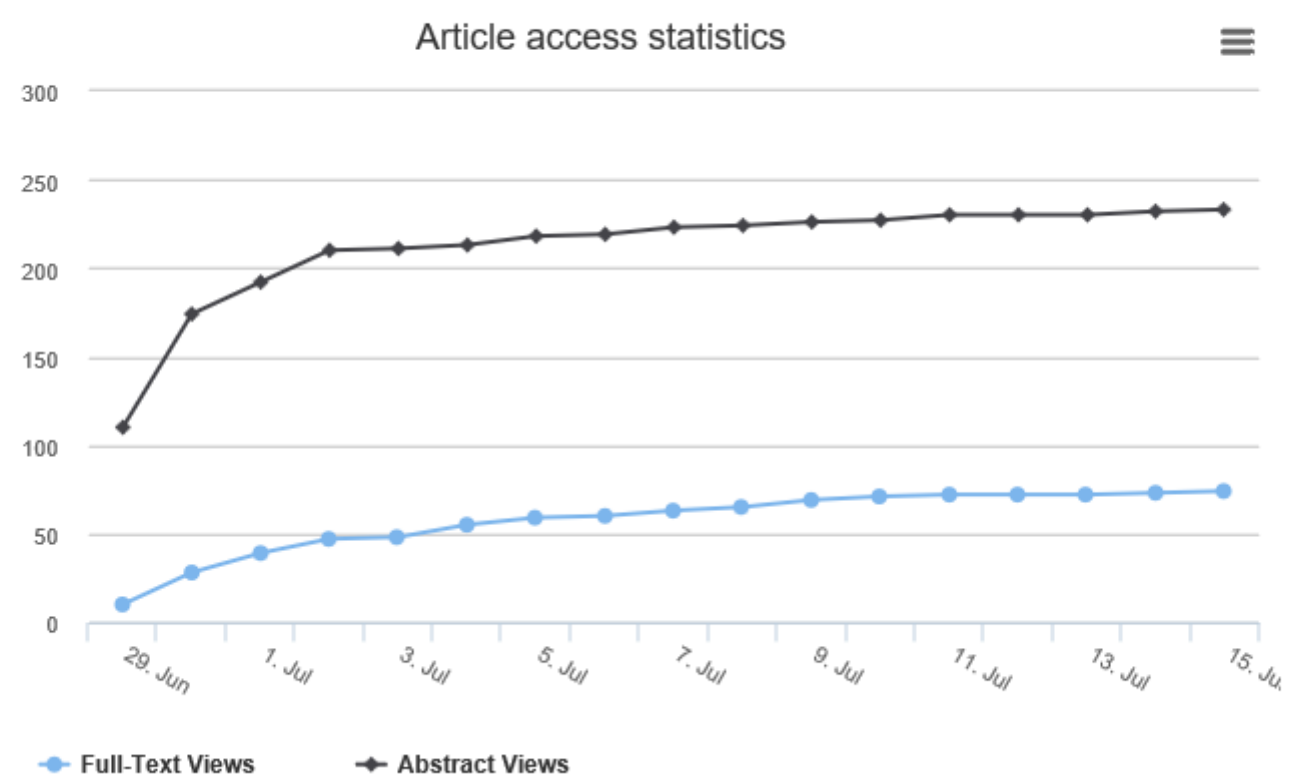


1. Introduction

CCAAT/enhancer-binding proteins (C/EBPs) are transcription factors that activate the expression of target genes through interaction with response elements within their promoters [1]. There are six members of C/EBP family, and they regulate differentiation process in various tissues [2]. The first transcription factor in this family, C/EBPα, was isolated from the rat liver and it appeared to be important for adipocyte differentiation [3]. C/EBPs are modular proteins consisting of an activation domain, a DNA binding domain, and a leucine-rich dimerization domain that is responsible for forming dimers with other members of the family [4]. In order to activate transcription, the C/EBP dimers bind to the consensus sequence 5′-TT/GNNGNAAT/G-3′ in promoter regions of target genes. For three out of six genes encoding C/EBP family members, alternative protein products are translated, due to a leaky ribosomal scanning mechanism. Some of them lack the N-terminal activation domains and exert inhibitory functions, presumably by a dominant negative mechanism [5].

Hematopoiesis is a process in which all blood elements are formed from multipotential hematopoietic stem cells (HSCs). In the process of hematopoiesis, the HSCs and their progeny

interact with the bone marrow stromal cells and they are stimulated by the numerous growth factors that are secreted in the bone marrow environment. The eventual cell fate during hematopoiesis is governed by spatiotemporal fluctuations in transcription factor concentrations, which either cooperate or compete in driving target gene expression [6]. Some members of C/EBP family of transcription factors are important at certain steps of hematopoiesis [7]. C/EBPα appears in differentiating cells at the stage of early progenitors with lymphoid and myeloid potential and then reappears only in the cells that are differentiating into granulocytes [8]. C/EBPα-deficient mice show disturbances in monocyte and neutrophil development [9,10]. High level of C/EBPβ leads to monocyte and macrophage development [11,12], while high level of C/EBPε leads to neutrophil differentiation [13]. The role of C/EBPδ in blood cells development is less defined, since *CEBPD*−/− mice did not reveal any blood disturbances [14]. It has been documented that C/EBPδ regulates expression of genes important for granulocyte function [15].

However, the most important factors that drive blood cells development are cytokines [16], some ligands for nuclear receptors can also modulate cell fate during hematopoiesis [7]. The best described in this respect are ligands for retinoic acid receptors (RARs). Active metabolites of vitamin A are natural ligands for RARs. A dominating retinoic acid (RA) metabolite is all-*trans*-RA (ATRA), which binds with high affinity to all RARs (α, β, and γ) [17]. During embryogenesis, ATRA causes the appearance of hematopoietic progenitors from the hemogenic endothelium [18], while in adults, it is important for the differentiation of granulocytes, as well as B and T lymphocytes [19]. This activity of ATRA has been used in clinics. The most clinically significant application of ATRA is to treat a rare subtype of an acute myeloid leukemia (AML), called acute promyelocytic leukemia (APL). At the first description this subtype was considered the most difficult to treat [20], while it is now considered as highly curable using the combination of ATRA and anthracycline-based chemotherapy [21]. Another ligand for the

using the combination of ATRA and anthracycline-based chemotherapy [21]. Another ligand for the nuclear receptor which influences hematopoiesis is an active metabolite of vitamin D. The correct physiological concentrations of 1,25-dihydroxyvitamin D (1,25D), which is a natural ligand for vitamin D receptor (VDR), are necessary to induce markers of monocytic differentiation in HSCs [22]. The expression of the *VDR* gene is higher at the early steps of hematopoiesis than at later stages and in mature blood cells [23]. However, both these ligands do not seem indispensable for blood cells development since RARα-deficient and VDR-deficient mice show no defects in hematopoiesis [24,25]. The possibility that these nuclear receptors can, in some aspects, functionally compensate each other should be considered.

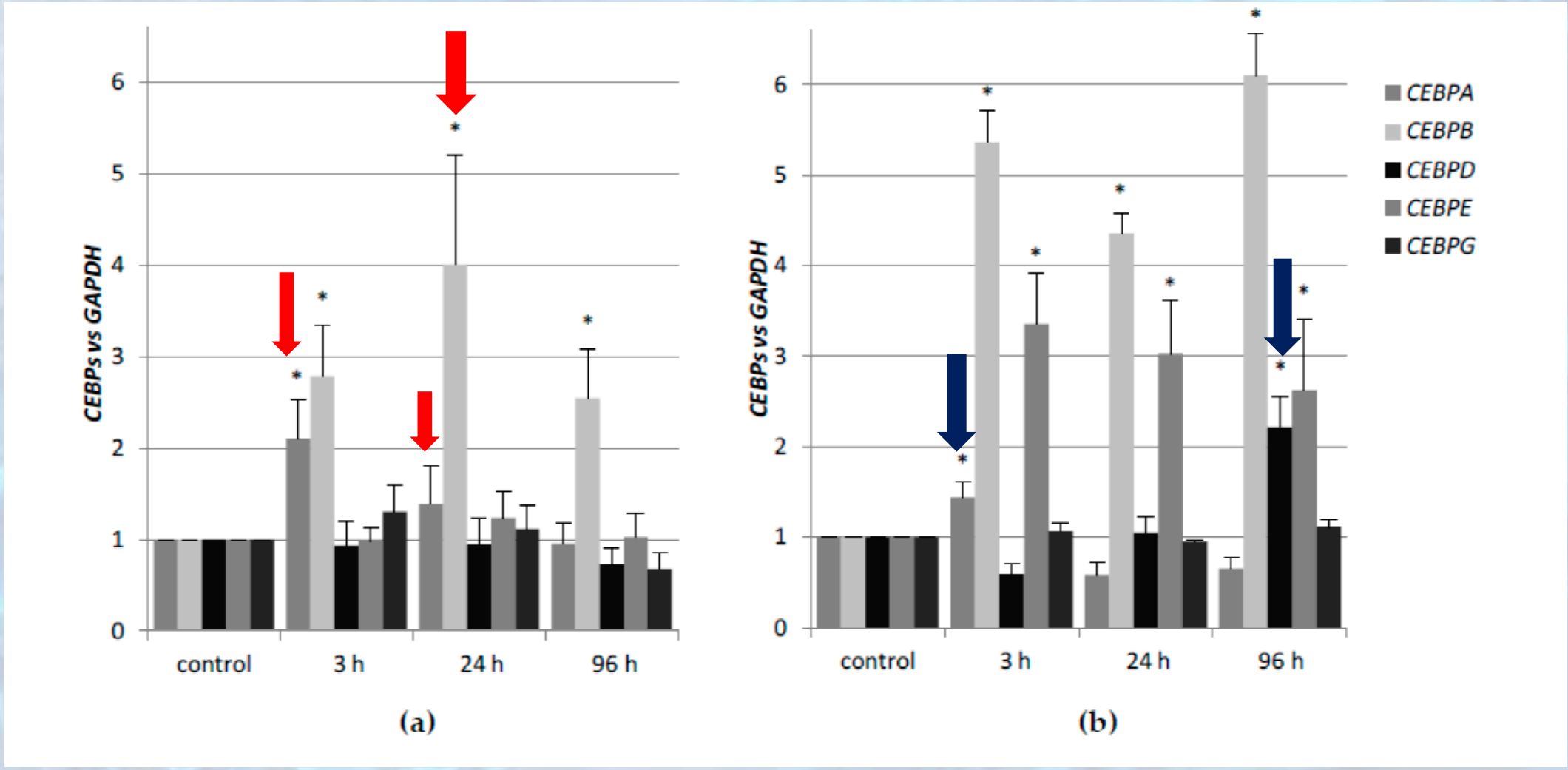
It has been documented in the past that members of C/EBP family of transcription factors can be upregulated in blood cells by an exposure to RA, 1,25D, or to their active analogs. For example, the expression of C/EBPε mRNA and protein increases in AML cells exposed to 9-*cis*–RA or 1,25D analog (KH1060) [26]. The gene encoding C/EBPβ has been shown to be a target for VDR regulation [27] and all isoforms of this transcription factor are increased in AML cells exposed to 1,25D or to analogs of 1,25D [11,28]. This gene is also strongly upregulated in AML cells that were exposed to ATRA [29]. 1,25D induces a transient increase of C/EBPα [11], which also participates in the ATRA-induced differentiation of AML cells [30].

In this study, we addressed a question of whether the lack of one of the nuclear receptors mentioned above could be compensated by the other in terms of *CEBP* activation. Therefore, we used four cell lines in our study, with different expression of retinoic acid receptor α (*RARA*) or *VDR*. In HL60 cells, *VDR* expression is on a high level and *RARA* is moderate [31]. For the purpose of this study, we silenced the expression of *VDR* in HL60 cells using shRNA. In contrast to HL60 cells, KG1 cell express high levels of *RARA*, but low of *VDR* [31]. The effects of *RARA* silencing were studied using a sub-line KG1-RARα(−).

2. Results

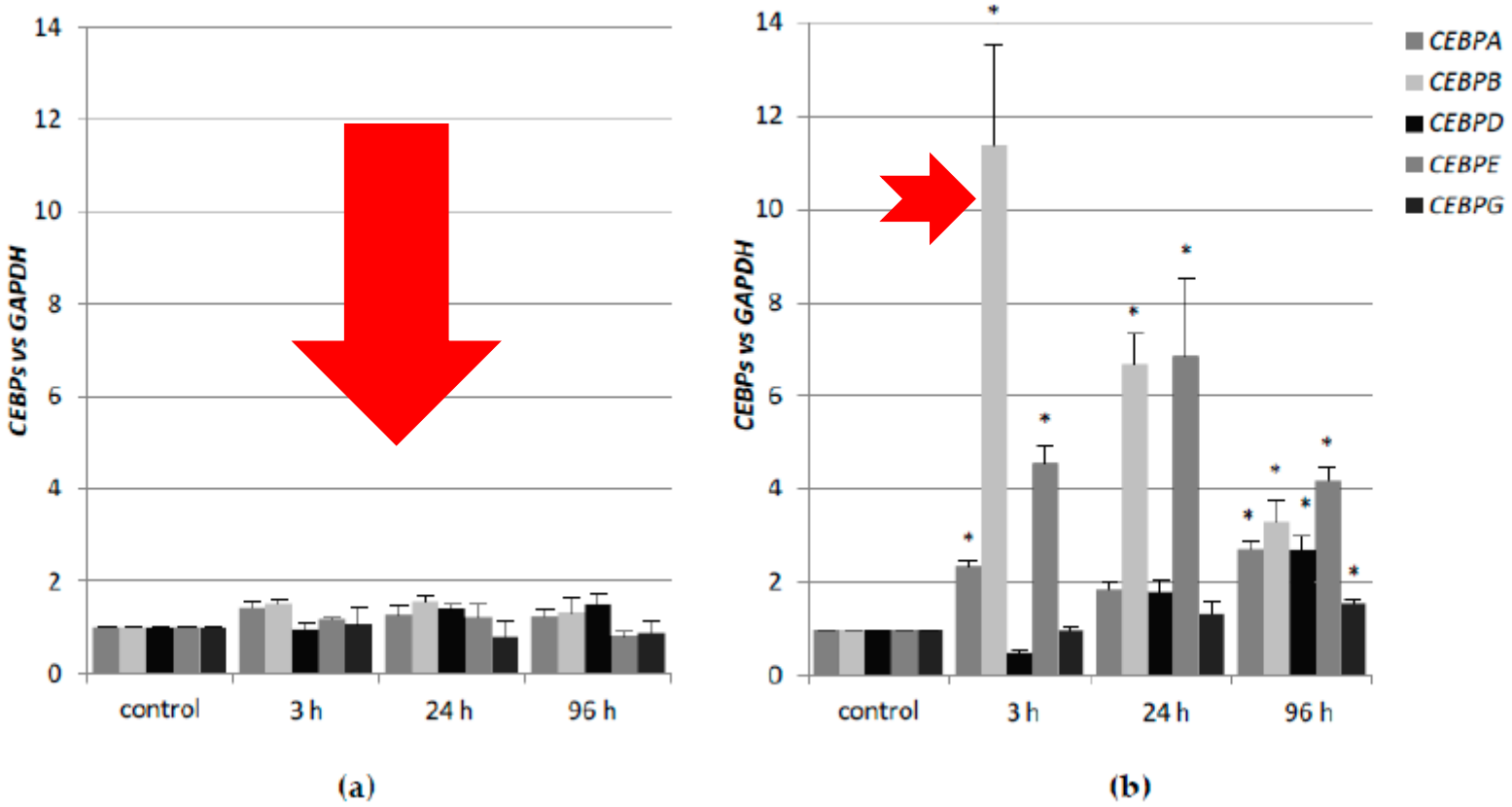
2.1. Activation of Expression of CEBP Transcription Factor's Genes in AML Cells with High Level of VDR and Low Level of RARα

In previous studies, we have shown that different AML cell lines have variable sensitivity to 1,25D– and ATRA-induced differentiation [32]. HL60 cell line responded to 1,25D with robust monocytic differentiation and to ATRA with moderate granulocytic differentiation. That corresponded to high basal level of expression of *VDR* and low basal level of expression of *RARA* [31]. In view of demonstrated regulation of differentiation of myeloid leukemia cells by these two compounds, it was of interest to determine the expression profiles of *CEBP* genes in response to 1,25D and ATRA in HL60 cells. Therefore, the expression of *CEBPA*, *CEBPB*, *CEBPD*, *CEBPE*, and *CEBPG* in HL60 cells that were exposed to 1 μM ATRA or to 10 nM 1,25D for different time periods was tested. As depicted


CEBPs vs GAPDH
0
1
2
3
4
5
6
control
3 h
24 h
96 h
(a)
CEBPA
CEBPB
CEBPD
CEBPE
CEBPG
(b)

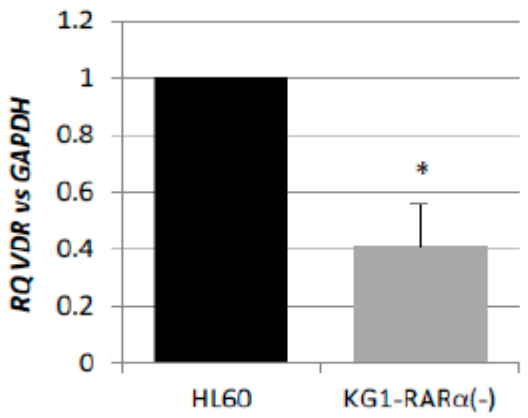
2.2. Activation of Expression of CEBP Transcription Factor's Genes in AML Cells with Low Level of VDR and High Level of RARα

In contrast to HL60 cells, KG1 cells are not responsive to 1,25D and they have a low level of VDR protein, whilst being susceptible to ATRA-driven granulocytic differentiation [33]. This corresponds with the high basal level of expression of *RARA* gene and high constitutive content of RARα protein [31]. In the next series of experiments, KG1 cells were exposed to 1 μM ATRA or to 10 nM 1,25D for different time periods. In KG1 cells, the transcript levels of *CEBP* genes remained unchanged after exposure to 1,25D (Figure 2a). In contrast, significant changes in expression of *CEBP* genes after exposure of KG1 cells to ATRA were observed. Modest upregulation of *CEBPA* was detected at 3 h and 96 h from exposure. *CEBPB* was the most responsive to ATRA out of the genes studied, the expression upregulation was fast and long-lasting. The second ATRA-responsive gene was *CEBPE*, where the expression peaked at 24 h. The expression of *CEBPD* and *CEBPG* was modest with a peak at 96 h (Figure 2b). Values of mRNA expression that were obtained using comparative quantification algorithm are presented in Table A2.

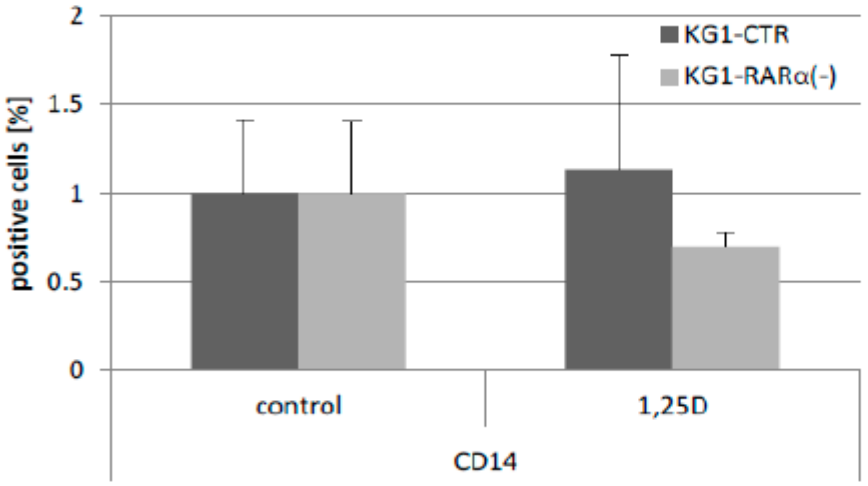

14
12
10
8
6
4
2
0
CEBPs vs GAPDH
control
3 h
24 h
96 h
(a)
(b)
CEBPA
CEBPB
CEBPD
CEBPE
CEBPG

2.3. Effects of Silencing High RARA on Expression of CEBP Transcription Factor's Genes in KG1 Cells

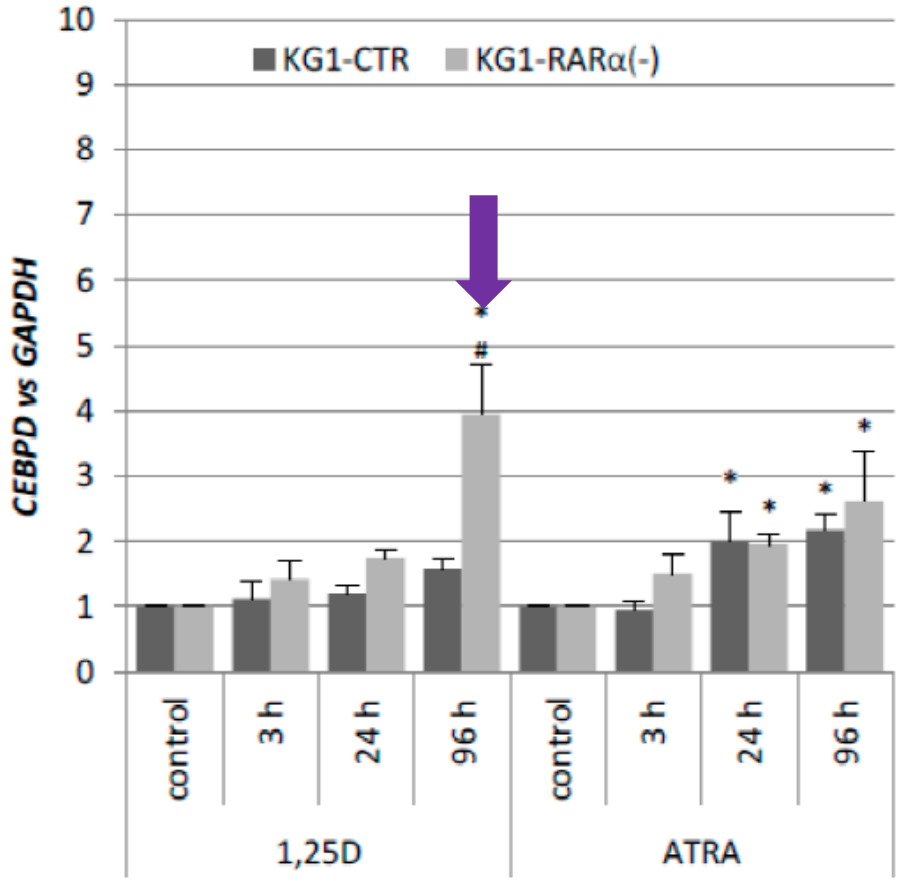
In an attempt to elucidate whether the lack of one of the nuclear receptors VDR and RARα could be compensated by the other in terms of *CEBP* activation, we used KG1 sublines with silenced *RARA* gene (KG1-RARα(−)) and KG1 control cells (KG1-CTR), which were obtained before [31]. These cells have substantially reduced level of *RARA* gene expression and RARα protein, but also exhibit the increased expression of *VDR* gene and VDR protein, when compared to wild-type KG1 and KG1-CTR [31]. It should be noted that the expression of *VDR* gene in KG1-RARα(−) is still lower than in HL60 cells (Figure 3a), and it was not sufficient to induce antigen CD14 typical for monocytes



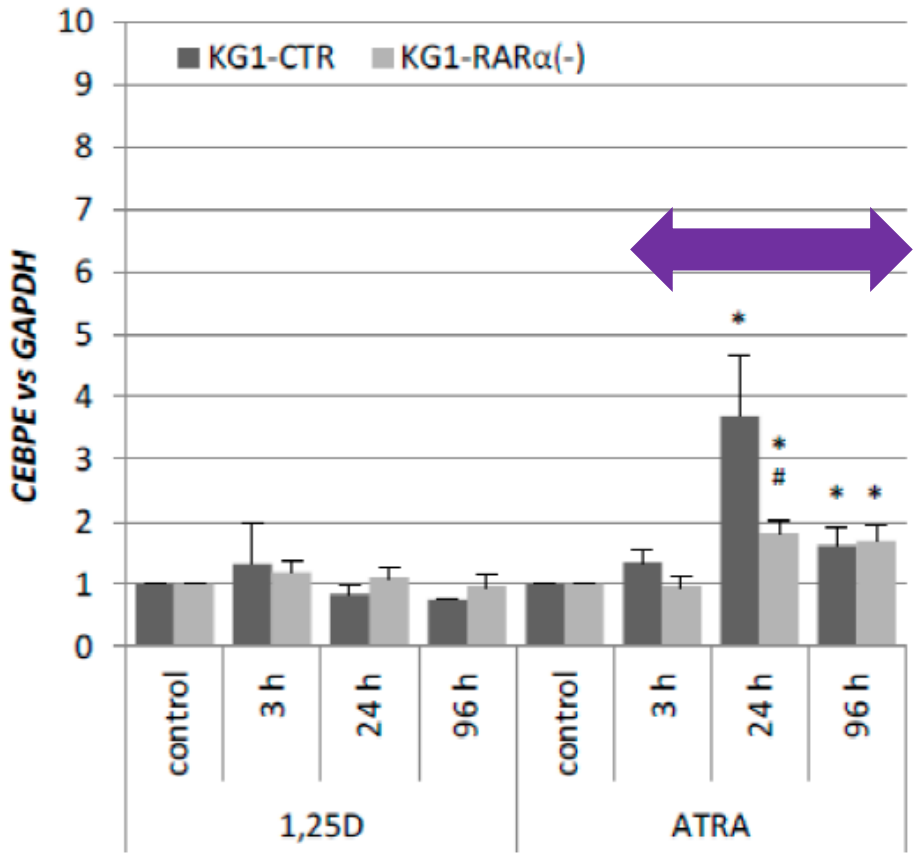
(a)



(b)

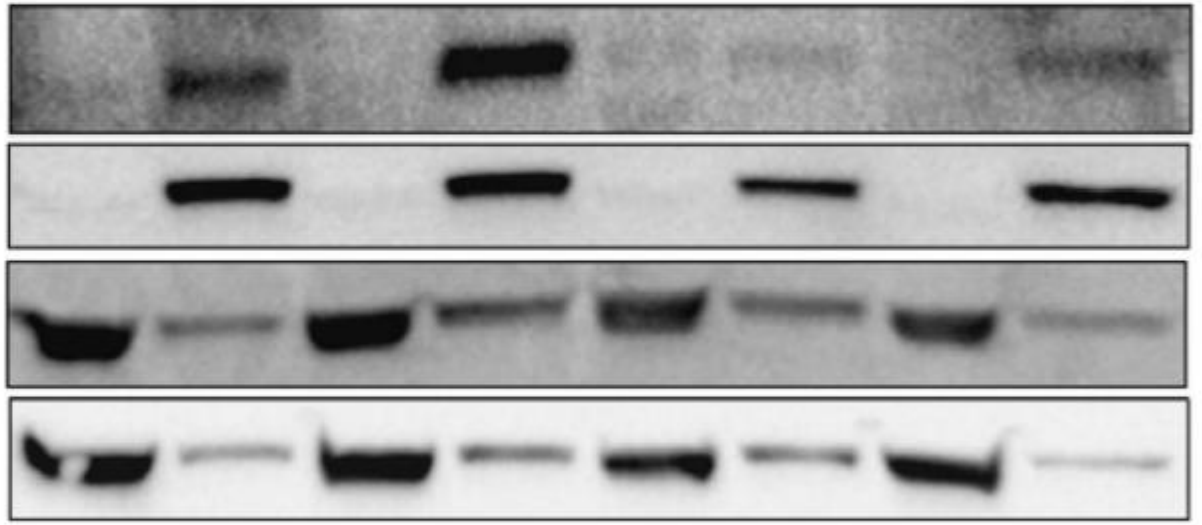

KG1-CTR
KG1-RARα(-)
CEBPD vs GAPDH
10
9
8
7
6
5
4
3
2
1
0
control
3 h
24 h
96 h
control
3 h
24 h
96 h
1,25D
ATRA

(e)


KG1-CTR
KG1-RARα(-)
CEBPE vs GAPDH
10
9
8
7
6
5
4
3
2
1
0
control
3 h
24 h
96 h
control
3 h
24 h
96 h
1,25D
ATRA

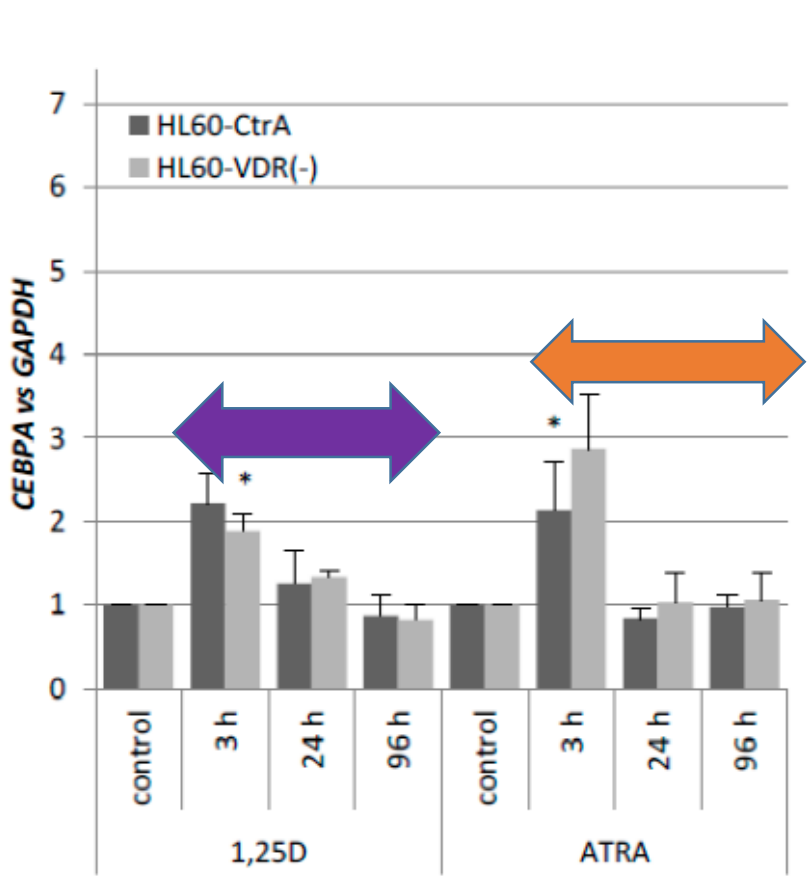
(f)

cells	HL60-CtrA				HL60-VDR(-)			
treatment	none		10nM 1,25D		none		10nM 1,25D	
fraction	C	N	C	N	C	N	C	N

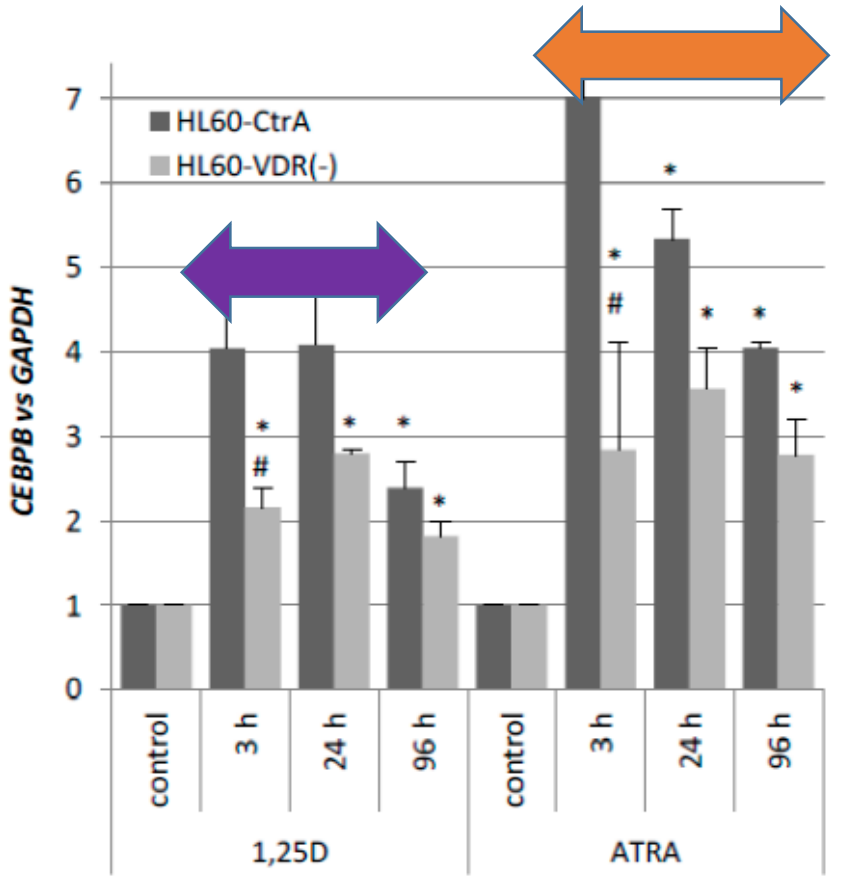


VDR/Actin	1.00	5.97	1.05	7.94	0.67	4.61	0.58	3.08 #
±SEM	0.0	2.77	0.57	4.28	0.49	1.05	0.18	1.82

(c)


HL60-CtrA
HL60-VDR(-)
CEBPA vs GAPDH
0
1
2
3
4
5
6
7
control
3 h
24 h
96 h
control
3 h
24 h
96 h
1,25D
ATRA

(a)


HL60-CtrA
HL60-VDR(-)
CEBPB vs GAPDH
0
1
2
3
4
5
6
7
control
3 h
24 h
96 h
control
3 h
24 h
96 h
1,25D
ATRA

(b)

It has been shown that both 1,25D and ATRA are able to upregulate expression of C/EBP factors without the addition of hematopoietic cytokines [11,26,27,29]. Whether all the *CEBP* genes are direct targets for either RARα or VDR is not clear. Retinoic acid response elements (RAREs) have been found in the promoter of *CEBPE* gene, and not in other genes of this family [44], but at present, we know that RARα can bind to big variety of RAREs [45], which are sometimes located in a long distance from the transcription start [44]. *CEBPA* and *CEBPB* are direct targets of VDR-dependent transcriptional regulation [36,37], but it is not sure whether such a mechanism occurs also for *CEBPD*.

Our results suggest that the ability of 1,25D or ATRA to effectively force the final myeloid differentiation of AML cells strongly depends on effective levels of nuclear receptors for these compounds. It also seems that expression of *CEBPB* is indispensable for the final effect of myeloid differentiation, and that VDR and RARα do not compensate each other in terms of the induction of *CEBP* expression. Our data are in agreement with the earlier findings that strong and sustained expression of *CEBPB*, when accompanied by transient expression of *CEBPA* leads to the differentiation towards monocytes [11], while, when accompanied by the sustained expression of *CEBPE*, it leads the differentiation process to granulocytes [46].


Thank You